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14. ABSTRACT
Both laboratory and epidemiological studies published over the past two decades have identified the risk of excess hearing loss when specific chemical contaminants are present along with noise. The objective of this study was to evaluate the potency of JP-8 jet fuel to enhance noise-induced hearing loss (NIHL) using inhalation exposure to fuel and simultaneous exposure to either continuous or intermittent noise exposure over a 4-wk exposure period using both male and female Fischer 344 rats. In the initial study, male (n = 5) and female (n = 5) rats received inhalation exposure to JP-8 fuel for 6 h/d, 5 d/wk for 4 wk at concentrations of 200, 750, or 1500 mg/m^3. Parallel groups of rats also received nondamaging noise (constant octave band noise at 85 dB_{lin}) in combination with the fuel, noise alone (75, 85, or 95 dB), or no exposure to fuel or noise. Significant concentration-related impairment of auditory function measured by distortion product otoacoustic emissions (DPOAE) and compound action potential (CAP) threshold was seen in rats exposed to combined JP-8 plus noise exposure when JP-8 levels of 1500 mg/m^3 were presented with trends toward impairment seen with 750 mg/m^3 JP-8 + noise. JP-8 alone exerted no significant effect on auditory function. In addition, noise was able to disrupt the DPOAE and increase auditory thresholds only when noise exposure was at 95 dB. In a subsequent study, male (n = 5 per group) and female (n = 5 per group) rats received 1000 mg/m^3 JP-8 for 6 h/d, 5 d/wk for 4 wk with and without exposure to 102 dB octave band noise that was present for 15 min out of each hour (total noise duration 90 min). Comparisons were made to rats receiving only noise, and those receiving no experimental treatment. Significant impairment of auditory thresholds especially for high-frequency tones was identified in the male rats receiving combined treatment. This study provides a basis for estimating excessive hearing loss under conditions of subchronic JP-8 jet fuel exposure.

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SUBCHRONIC JP-8 JET FUEL EXPOSURE ENHANCES VULNERABILITY TO NOISE-INDUCED HEARING LOSS IN RATS

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Both laboratory and epidemiological studies published over the past two decades have identified the risk of excess hearing loss when specific chemical contaminants are present along with noise. The objective of this study was to evaluate the potency of JP-8 jet fuel to enhance noise-induced hearing loss (NIHL) using inhalation exposure to fuel and simultaneous exposure to either continuous or intermittent noise exposure over a 4-wk exposure period using both male and female Fischer 344 rats. In the initial study, male ($n = 5$) and female ($n = 5$) rats received inhalation exposure to JP-8 fuel for 6 h/d, 5 d/wk for 4 wk at concentrations of 200, 750, or 1500 mg/m^3. Parallel groups of rats also received nondamaging noise (constant octave band noise at 85 dB_{lin}) in combination with the fuel, noise alone (75, 85, or 95 dB), or no exposure to fuel or noise. Significant concentration-related impairment of auditory function measured by distortion product otoacoustic emissions (DPOAE) and compound action potential (CAP) threshold was seen in rats exposed to combined JP-8 plus noise exposure when JP-8 levels of 1500 mg/m^3 were presented with trends toward impairment seen with 750 mg/m^3 JP-8 + noise. JP-8 alone exerted no significant effect on auditory function. In addition, noise was able to disrupt the DPOAE and increase auditory thresholds only when noise exposure was at 95 dB. In a subsequent study, male ($n = 5$ per group) and female ($n = 5$ per group) rats received 1000 mg/m^3 JP-8 for 6 h/d, 5 d/wk for 4 wk with and without exposure to 102 dB octave band noise that was present for 15 min out of each hour (total noise duration 90 min). Comparisons were made to rats receiving only noise, and those

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The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996.

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receiving no experimental treatment. Significant impairment of auditory thresholds especially for high-frequency tones was identified in the male rats receiving combined treatment. This study provides a basis for estimating excessive hearing loss under conditions of subchronic JP-8 jet fuel exposure.

Laboratory investigations have identified a variety of chemicals of both occupational and environmental interest that are capable of producing hearing loss. The relevance of such data for human occupational exposure has been questioned at times because, in general, exceedingly high dose levels relative to permissible exposure levels (PEL) established for human occupational exposures and protracted exposure times are needed for ototoxicity to be observed. For example, toluene ototoxicity in rats was seen at exposure levels between 1000 and 2000 ppm over 3–5 d with 1300 ppm seen as a threshold dose for permanent hearing loss when exposures of 4 wk are utilized (Crofton et al. 1994; Johnson and Canlon 1994; Pryor et al. 1983; Rebert et al. 1983; Sullivan et al. 1988). Ethylbenzene ototoxicity was observed at exposure concentrations of 300–400 ppm for 5 d (Cappaert et al. 2000). Maguin et al. (2006) found *p*-xylene produced ototoxicity at exposure at levels of 1800 ppm for 3 wk. The Occupational Safety and Health Administration (OSHA) has set the PEL for toluene at 200 ppm, and for xylenes and ethylbenzene at 100 ppm. The American Conference of Government Industrial Hygienists (ACGIH) recommended a threshold limit value (TLV) of 50 ppm for toluene, and 100 ppm for xylenes and ethylbenzene. However, it has also been documented in laboratory animals that ototoxicity may be observed at more realistic exposure concentrations if the subjects are required to be active during exposure, rather than being sedentary (Lataye et al. 2005). In addition, ototoxicity may be observed at lower exposure concentrations if noise is also present in the environment (Fechter 2004; Fechter et al. 2000). The finding of an interaction between the effects of noise and chemical agents on hearing loss is particularly problematical given that in most instances occupational exposure levels are established based upon exposures to a single agent rather than to combined exposures to chemical and physical agents. In only a few rare exceptions, for example, does the ACGIH recommend that auditory testing be pursued more aggressively if select chemicals are present in noisy environments (ACGIH 2002).

This investigation was undertaken to determine the ototoxic potential of subchronic JP-8 jet fuel both by itself and in the presence of either continuous or interrupted noise. JP-8 is a traditional petroleum-derived fuel that is closely related to Jet A fuel used in commercial aviation. Both of these aviation fuels contain aromatic hydrocarbons (25% maximum). JP-8, designated as MIL-DTL-83133, has become the standard fuel used by the U.S. Armed Services and by NATO. Several of the aromatic hydrocarbons contained in JP-8 fuel are known to be ototoxic based upon both epidemiological (Abbate et al. 1993; Morata et al. 1997; Vrca et al. 1996; 1997; Sliwinska-Kowalska, et al. 2001; 2003; Fuente et al. 2009) and controlled laboratory studies (Campo et al. 1997; 2001; Cappaert et al. 1999; 2000; 2001a; 2001b; Crofton et al. 1994; Loquet et al. 1999; Pryor et al. 1983; 1987; McWilliams et al. 2000; Lataye et al. 2003; Gagnaire and Langlais 2005).

There have been prior investigations on the effects of jet fuel on hearing in occupational settings, as well as short-term studies among laboratory subjects. Kaufman et al. (2005) studied a small sample of U.S. Air Force employees ($n = 48$ exposed, 90 unexposed) with occupational exposure to noise and jet fuels (JP-4 and JP-8) containing aromatic hydrocarbons and reported that jet fuel may increase hearing loss in a chronic exposure model (a larger odds ratio [OR]) for hearing loss with 12 yr of exposure versus for 3 yr of exposure. Moreover, the OR associated with duration of fuel exposure exceeded that obtained for age. However, since all subjects did have a history of noise exposure,

it is not clear whether the fuel by itself might have produced some ototoxicity. There is a high probability for combined exposure to jet fuel and to noise in a wide range of occupations related to airplane operations, including maintenance workers, aircrews, and fuel deliverers (Kaufman et al. 2005; Owen 1996; Ritchie et al. 2001).

Fechter, et al. (2007; 2010) reported that subacute exposures in rats to JP-8 jet fuel by itself exerted no marked effect on auditory function up to concentrations of 2000 mg/m^3, assessed either using distortion product otoacoustic emissions (DPOAE) or on pure tone auditory thresholds assessed by measuring the occurrence of a compound action potential (CAP). However, exposure to JP-8 enhanced the adverse effects of moderate noise exposure on DPOAE amplitude. Specifically, successive exposure first to JP-8 jet fuel (1000 mg/m^3 for 4 h/d $\times$ 5 d) followed on each of the 5 d by a 1-h exposure to 100 dB_{lin} octave band noise (OBN) yielded a persistent reduction in the DPOAE of 10–20 dB. The noise exposure alone produced minimal impairments on this measure of outer hair cell (OHC) function.

The current study used a more appropriate design for evaluating the combined effects of JP-8 and noise exposure in that noise and fuel exposures occurred simultaneously over a longer time period each day and over a 4-wk duration (5 d/wk for 4 wk). This study required the identification of a noise exposure protocol that yielded the lowest-observed-adverse-effect level (LOAEL) on hearing, a concentration response study to identify a LOAEL and no-observed-adverse-effect level (NOAEL) for JP-8 jet fuel by itself, and the characterization of JP-8 exposure able to increase susceptibility to noise-induced hearing loss (NIHL). Once these objectives were met using a continuous noise exposure paradigm, an additional study was undertaken to determine the efficacy of JP-8 to promote NIHL induced by an intermittent noise exposure, since intermittent noise is a far more common workplace experience than is continuous noise over the course of a work day.

METHODS AND MATERIALS

Subjects

For all studies, Fischer 344 (F344) male and female rats obtained from Charles River Laboratories (Wilmington, MA) were employed as subjects. The rats were purchased at approximately 6-8 wk of age. The males averaged 119 g body weight (bw) while female rats weighed approximately 100 g for the initial 2 experiments described here. Because of their small size and susceptibility to anesthetic overdose, larger female rats weighing 146 g on average were ordered for the last 2 experiments described here. All of the rats were initially housed at Wright-Patterson Air Force Base (WPAFB), Dayton, OH. Seven days following their arrival, the rats received a brief assessment of auditory function in order to equate auditory function across all groups and then received their assigned noise and JP-8 exposure (described later). The rats did not receive further assessments of auditory function at WPAFB since the focus of this study was on permanent auditory impairment rather than the transient effects commonly observed following noise exposure. Three days following the conclusion of experimental exposures, the rats were transported by temperature controlled vans and commercial airplane to the Jerry Pettis Memorial VA Medical Center in Loma Linda, CA (LLVAMC), where they received extensive auditory testing and were ultimately euthanized, with cochleae harvested for assessment of inner ear pathology. The subjects were housed in plastic cages with free access to food and water. Temperature was maintained at 21 $\pm$ 1°C and fluorescent lights were on from 6:30 a.m. to 6:30 p.m. All procedures used were approved by the Institutional Animal Care and Use Committees (IACUC) both at WPAFB and at the LLVAMC. All exposures and testing were performed during the daytime.

Exposure Procedures

Because of limitations posed by the number of inhalation chambers available (a total of four chambers), a series of studies was conducted in which (a) an appropriate continuous

TABLE 1. Summary of Treatments

Experiment	Males (*n*)	Females (*n*)
Noise dose response		
75 dB OBN 6 h/d, 5 d/wk for 28 d	5	5
85 dB OBN 6 h/d, 5 d/wk for 28 d	5	5
95 dB OBN 6 h/d, 5 d/wk for 28 d	5	5
Controls	5	5
JP-8 versus JP-8 + continuous noise		
JP-8 200 mg/m^3 6h/d, 5 d/wk for 28 d	5	5
JP-8 750 mg/m^3 6h/d, 5 d/wk for 28 d	5	5
JP-8 1500 mg/m^3 6h/d, 5 d/wk for 28 d	5	5
Controls	5	5
JP-8 200 mg/m^3 + 85 dB OBN 6 h/d, 5 d/wk for 28 d	5	5
JP-8 750 mg m^3 + 85 dB OBN 6 h/d, 5 d/wk for 28 d	5	5
JP-8 1500 mg/m^3 + 85 dB OBN 6 h/d, 5 d/wk for 28 d	5	5
Controls	5	5
JP-8 + intermittent noise		
JP-8 1000 mg/m^3 6 h/d, 5 d/wk for 28 d	5	5
JP-8 1000 mg/m^3 6 h/d, 5 d/wk for 28 d + 102 dB intermittent OBN 90 min/day total	5	5
102 dB intermittent OBN totaling 90 min/day, 5 d/wk for 28 d	5	5
Controls	5	5

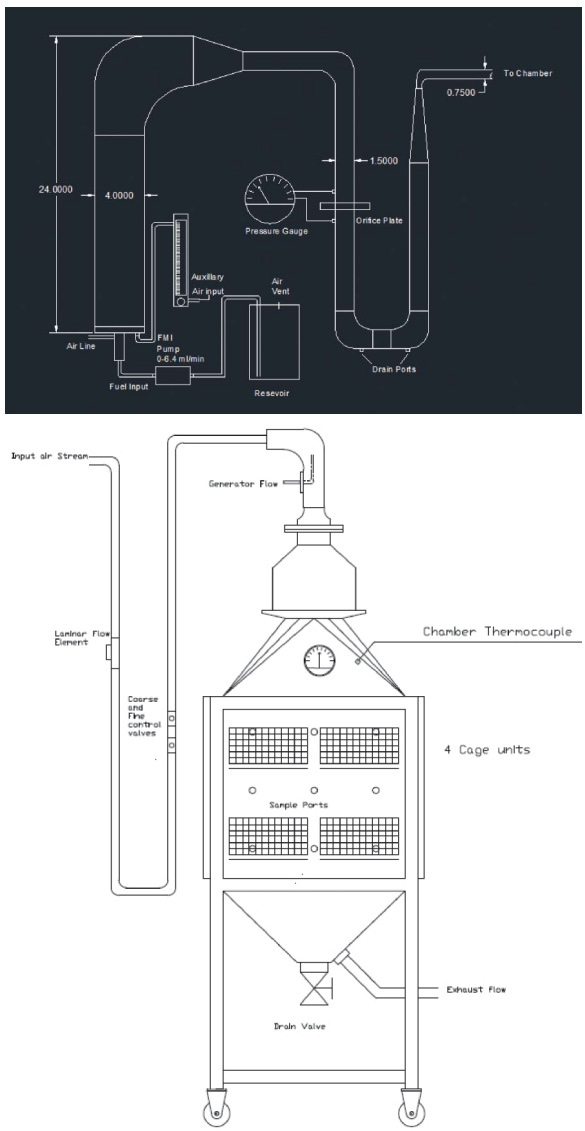

FIGURE 1. Schematic representation of (A) JP-8 jet fuel generation system for inhalation exposure and (B) whole body exposure chambers.

noise exposure level was determined (see later description), (b) a concentration-response study for the effect of JP-8 alone on auditory function was conducted, and (c) the effects of simultaneous continuous noise and JP-8 exposure were determined. Finally, a study of the interaction of JP-8 jet fuel and intermittent noise exposure was undertaken. Table 1 summarizes the exposure treatments used in each experiment, and Figure 1 provides a summary of the jet fuel generation system.

Jet Fuel Three different concentrations of jet fuel were used in the initial study (200, 750, and 1500 mg/m^3 total hydrocarbon levels), which bracketed the concentration previously shown to promote NIHL using a nose only exposure for 5 d (Fechter et al. 2007; 2010). For comparison, the permissible concentration for JP-8 in the workplace is 200 mg/m^3 based on an 8-h time-weighted average (TWA). Half of the rats receiving jet fuel also received noise exposure as described later.

The fuel was supplied from a stock maintained by the Fuels Branch (AFRL/RZPF) at WPAFB (Dayton, OH). It consisted of a blend of jet fuel obtained from various refineries, to which the JP-8 fuel additive package, consisting of diethylene glycol monomethyl ester to inhibit ice formation and both static and corrosion inhibitors, was added. A single lot of fuel was used to complete all of the studies described here.

The fuel generation system for the high concentration of 1500 mg/m^3 is shown in Figure 1. Jet fuel was pumped from a reservoir using a FMI model QG20 pump with a Q1CKC pump head (FMI, Inc., Syosset, NY) into the fuel

input port of a Sonimist ultrasonic spray nozzle (model HSS600-2, Misonix, Inc., Farmingdale, NY). An air line set to 40 pounds per square inch (psi) pressure was attached to the side arm of the Sonomist. At this pressure the spray nozzle developed an air flow of approximately 20 L/min (lpm) through the nebulizer. This air flow coupled with the nebulizer nozzle design created an ultrasonic whistle that aerosolized the droplets of jet fuel being formed at the end of the nozzle and acted as a carrier for the jet fuel into the generating system. A 2-foot length of 4-inch PVC pipe contained the spray pattern. The pipe was reduced in size to accept an orifice plate, which was used to measure flow rate by the pressure drop across the plate. The pipe diameter was reduced one final time to 3/4 inch and the aerosolized jet fuel was transported to the chamber, where it was injected as a countercurrent into the main chamber flow. Two drain ports were used to remove residual jet fuel that accumulated after a day's exposure. To achieve the 1500-mg/m^3 concentration, the high-concentration generation system used an HSS600-2 nebulizer, which has greater throughput and did not develop problems with fuel accumulation around the nebulizer. In addition, no orifice plate was used in the line. A small amount of excess fuel accumulated in the system during exposure at the drain ports. Adding auxiliary air kept the jet fuel accumulation to a minimum that did not interfere with flow.

The mid- and low-concentration generation systems used a Sonomist HSS600-1 nebulizer, no orifice plate, and a 0.5-inch line to the chamber. To eliminate problems with occasional nebulizer malfunction due to jet fuel accumulation around the nebulizer, the 4-inch pipe was inverted so the nebulizer aimed down rather than up as in Figure 1. The high-concentration generation system could not be inverted due to differences in the parts used to assemble that system. The mid-concentration system was still accumulating too much jet fuel in the lower parts where the drain ports were added, so auxiliary air was added to the mid-range system as well, which eliminated jet fuel accumulation.

The rats were exposed to JP-8 using a whole-body exposure system consisting of whole-body 690-L toxic hazard research units (THRU) chambers. Each chamber was operated with a total flow of 180 lpm consisting of the combination of jet fuel generator input and the main airflow. The main airflow was supplied by two Spencer vortex blowers (model VB030SB-012); one provided input air and one handled exhaust flow. The exhaust air flow was adjusted to maintain a slightly negative 1 to 2 inches of water below ambient pressure inside the chamber, as measured with a magnehelic pressure gauge (Dwyer Instruments, Champlain, NY) attached to the upper plenum of the chamber. Airflow through the chambers was controlled with mechanical valves, which were adjusted to obtain the desired flow rate. Flow rate was monitored on the input side of the chamber using a Hastings (model LSD58D, Teledyne-Hastings, Hampton VA) laminar flow unit, and the signal was monitored using a Hastings (model 40) monitor. The back of the chamber has nine ports, which can be used for various sampling devices. Attached to one port was a Nicolet (Thermo Scientific, model IS10) Fourier-transform infrared spectrophotometer (FTIR) equipped with either a 2-m path length gas cell for high concentrations or a 10-m path length gas cell for lower concentrations. Prior to entering the FTIR, the aerosol portion of the sample was removed using a small HEPA filter. Sampling by the FTIR was controlled using a macro on a computer that averaged every 10 spectrums collected, displayed the average concentration of jet fuel on the screen, and saved the data to a file. The system was programmed to collect and save one sample per minute for the entire 6-h exposure period.

Noise Exposure The noise exposure selected was designed to produce a just observable permanent impairment in auditory function, but one sufficient such that additive or potentiating effects of chemical exposure could also be detected (Pouyatos et al. 2005; Rao and Fechter 2000). The noise level used was an octave band (OBN) centered at 8 kHz so as to yield cochlear injury at frequencies

within the most sensitive portion of the rat's audiogram (approximately 8–20 kHz).

Current OSHA standards have established a PEL for noise of 90 dB using the A weighting scale for an 8-h TWA with an action level of 85 dB(A), at which point specific measures must be adopted to limit noise exposure. Audiograms are an annual requirement. A 5-dB exchange rate is utilized for intermittent noise and for noise that does not persist for 8 h. Based upon this rule, the equivalent human PEL for a 4-h time period would be 95 dB (A), and for 1 h, 105 dB (A). Notably, the exchange rule used in Europe is 3 dB rather than 5 dB for intermittent noise. In the initial noise study, exposure levels of 75, 85, and 95 dB were employed for time periods of 6 h. These noise levels have OSHA equivalents of approximately 72, 82, and 92 dB on an 8-h exposure basis. Thus, the noise employed in these studies would bracket the exposure limit permitted by OSHA for workplace exposures, with only the highest exposure level exceeding the human occupational PEL. Computer software installed on a laptop computer was used to generate a pure and precisely filtered white noise file. A high-pass filter with a 48 dB per octave roll-off was applied within the software to attenuate frequencies below 5.6 kHz, followed by a low-pass filter with the same roll-off value to attenuate frequencies above 11.3 kHz. The filter produced a finished file of one OBN, centered at 8 kHz. The filtered file was then played through electrodynamic shakers that induced vibration from the outside in the metal plenums at the bottom of each exposure chamber. During exposures, the sound intensity was measured inside the chambers at a central reference point using a Spectral Dynamics Puma data acquisition system (Spectral Dynamics, San Jose, CA). The system had four active input channels for monitoring and recording real-time sound levels in four chambers simultaneously. A 20-foot coaxial cable was connected to each of the output channels and a PCB model 378B20 1.27 cm (0.5 inch) random incidence microphone assembly was connected to the other end of each cable. A 1.27-cm (0.5-inch) inside diameter PVC pipe was installed through the center port on the rear of each chamber so the microphone could be positioned at the central reference point. Sound pressure measurements for chamber characterization were made using a Larson Davis model 831 sound level meter with a 6.1-m (20-foot) extension cable and microphone preamplifier. Distribution of sound pressure levels across 10 chamber exposure points were well controlled within ±1.5 dB. Stability at the central reference point was well controlled over 6-h runs within ±1 dB.

Following completion of an initial noise intensity study conducted with the purpose of assessing a NOAEL for noise alone and a study of auditory function within a JP-8 concentration-response study, two studies were completed in which the rats were assigned to receive noise exposure along with jet fuel inhalation while the remaining subjects received no experimental exposure (control).

Auditory Assessment

Outer hair cell (OHC) functional assessment: Distortion product otoacoustic emissionssions (DPOAE). Outer hair cell (OHC) function was assessed in subjects prior to any other experimental manipulation as a means of equating auditory function across treatment groups in a noninvasive manner. The DPOAE test relies upon the finding that the intact cochlea is able to generate measurable sound energy when stimulated with two simultaneous tones known as "primary tones" and designated as frequencies f_1 and f_2 (Kemp 1998). Because the sound energy generated by the cochlea consists of different frequencies than the primary tones, they are spoken of as "distortion products." A particularly robust distortion product is the cubic distortion product, which is defined algebraically as $2f_1 - f_2$. If the ratio of f_1/f_2 is kept constant as the frequency of f_2 is swept along the subject's audiometric range, it is possible to detect impairment of the hair cells as a drop in DPOAE amplitude. In these experiments, the ratio of f_1/f_2 was maintained at 1.25 and the f_2 frequency was swept from 3.197 to 19.401 kHz in the initial screening for auditory function. A more extensive evaluation of DPOAE amplitude was undertaken in all postexposure assessments. Here, the f_2 frequency was swept from 3.1 to 63 kHz in

0.1-octave increments. Tone intensities were set at 55 dB for f_1 and 35 dB for f_2. This difference in tone intensity was selected to maximize the amplitude of the DPOAE (Whitehead et al. 1995). The f_1 and f_2 primaries were presented through two separate realistic dual radial horn tweeters (Radio Shack, Tandy Corp., Ft Worth, TX). The tones were delivered to the outer ear canal through a probe that also contained an emissions microphone assembly (Etymotic Research, ER-10B+, Elk Grove Village, IL). The tones were sampled, synchronously averaged, and Fourier analyzed for geometric mean frequencies. Delivery of the primary test tones and computation of the $2f_1 - f_2$ distortion product amplitude were accomplished by a digital signal processor board (National Instruments model PCI-4461, Austin, TX) controlled by a dedicated program written using LabVIEW version 7.1 (National Instruments, Austin, TX). The related noise floors were estimated by averaging the levels of the ear-canal sound pressure for the two fast Fourier transform frequency bins below the DPOAE frequency (i.e., for 3.75 Hz below the DPOAE). A hard-walled cavity that approximated the size of the rat outer ear canal was used to calibrate the tonal stimuli. For both stimulus protocols, DPOAE were considered to be present when they were at least 3 dB above the noise floor.

DPOAE testing was accomplished in a single walled audiometric booth while rats were lightly anesthetized with ketamine (44 mg/kg body weight [bw]) and dexdomitor (0.25 mg/kg bw) injected intramuscularly (im). Normal body temperature was maintained using a direct current (dc) heating unit built into the table supporting the rat. To assess the effects of noise intensity alone on auditory function in the initial study, DPOAE amplitudes were assessed at only 1 postexposure time point, 4 wk, so that permanent impairment could be assessed. In subsequent studies, each subject was tested 10 d after the end of the experimental treatment, and again at 4 wk postexposure. Each DPOAE test required approximately 3 min to perform. The rats subsequently received a dose of atipamezole (0.1mg/rat) to reverse the anesthesia.

Audiometric threshold assessment: CAP. In contrast to the repeated noninvasive assessment of OHC function by the DPOAE method, assessment of auditory threshold, a marker of neural activity in the auditory branch of the eighth cranial nerve, requires nonsurvival surgery. Threshold assessment was performed 4 wk following the end of all experimental exposures by recording the CAP from the round window for pure tones between 2 and 40 kHz in approximately $\frac{1}{2}$-octave steps. The CAP is a marker of synchronous auditory nerve action potentials elicited by pure tone stimuli. Auditory thresholds were assessed in a double-walled audiometric booth. Preparation of subjects for CAP assessment required nonsurvival surgical procedures performed under anesthesia (75 mg/kg bw ketamine and 0.5 mg/kg bw dexdomitor). The auditory bulla was opened via a ventrolateral approach to allow the placement of a fine (outside diameter [OD] 0.1 mm) Teflon-coated silver wire electrode (A-M Systems, Inc., Carlsborg, WA) onto the round window. A silver chloride reference electrode was inserted into neck musculature. The cochlea was warmed using a low-voltage high-intensity lamp. Tonal stimuli were generated and shaped using a SoundMax Integrated Digital Audio board. A dedicated program running within LabVIEW 7.1 (National Instruments, Austin, TX) was used to control stimulus intensity, frequency, and timing. Each pure tone stimulus consisted of a 10-ms burst with 1-ms onset and offset ramps. Tones were presented at a frequency of 9.7/s. The computer program allowed tones to be augmented in 1-dB intensity steps until a discernable CAP was identified on a digital oscilloscope by the experimenter. The CAP signals evoked by pure tones were amplified $\times$1000 between 0.1 and 1.0 kHz with a Grass A.C. preamplifier (model P15, W. Warwick, RI). The sound level necessary to generate a visually detectable CAP response averaged over four sweeps on a digital oscilloscope (approximate response amplitude of 1 mV measured as the output of the preamplifier) was identified. Identification of the first negative wave of the action potential (N_1) response was based upon shape of the response, as well as

on its temporal relationship to the onset of the tonal stimulus. The CAP threshold was defined as the highest stimulus intensity at which the N_1 response was no longer observed against the noise background.

Histopathology In addition to the functional testing just described, subjects were euthanized at the conclusion of testing and cochleae were harvested for evaluation of hair cell death. Immediately after CAP measurements, rats were decapitated and the cochleae were harvested. Within 2 min, the cochleae were fixed by perilymphatic perfusion with 1 ml of a trialdehyde fixative (3% glutaraldehyde, 2% formaldehyde, 1% acrolein, and 2.5% dimethyl sulfoxide [DMSO] in phosphate-buffered saline, pH 7.4). Following the primary 24-h fixation, the tissue was first washed with 0.1 *M* phosphate-buffered saline, post-fixed with 2% osmium tetroxide in water for 2 h, and finally washed again with 0.1 *M* phosphate-buffered saline. The organ of Corti was dissected in 70% ethanol and mounted in glycerin to allow counting of the hair cells. Cells were counted as present either when the stereocilia, the cuticular plate, or the cell nucleus could be visualized. No attempt was made to assess the degree of possible cellular damage to surviving cells. The frequency-place map established by Muller (1991) was used to superimpose the frequency coordinates on the length coordinates of the organ of Corti. This "map" reflects the fact that the cochlea is organized in a tonotopic fashion, with high-frequency sound producing maximum stimulation of cells in the base, and low-frequency sound in the apex. A cochleogram showing the percentage of hair cell loss as a function of distance from the apex of the cochlea was plotted for each animal. The results were averaged within each group of subjects for comparison between groups.

Statistical Analysis

Separate split-plot factorial analysis of variance (ANOVA) tests were performed on the DPOAE amplitude data and the CAP threshold data using treatment and gender as between-subject variables and frequency as a repeated measure. In most instances, there were no gender differences, and in those instances only the combined data are presented and the variable gender was dropped from the statistical analyses. For the analyses of DPOAE data, the range of frequencies analyzed was 5.2–16.9 kHz as this corresponds to the frequencies that are susceptible to NIHL from an OBN centered at 8 kHz while eliminating the frequency range of approximately 20–25 kHz where instabilities occur in the DPOAE response due to outer ear canal resonance. A Greenhouse–Geiser correction was applied in all instances. Post hoc analyses were conducted using Bonferroni pairwise multiple comparisons. Results obtained with a p value $<.05$ are reported as statistically significant.

RESULTS

The effects of noise treatment alone on auditory function and structural integrity of the cochlea 4 wk following the noise exposure are portrayed in Figures 2–4. The distortion product test conducted 4 wk postexposure showed a reduction in DPOAE amplitude, indicative of OHC impairment, in a concentration-related manner within the anticipated frequency range

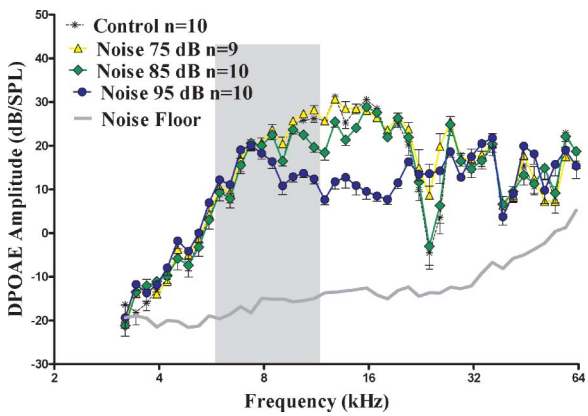

FIGURE 2. DPOAE amplitudes (mean ± standard error of the mean) among rats exposed to noise treatment alone at 75, 85, and 95 dB(A) for 6 h/d for 4 wk compared to untreated control subjects. The shaded area denotes the range of frequencies contained in the noise exposure. The ANOVA test demonstrated a significant effect of noise treatment ($F_{3/35} = 13.68$, $p < .0001$), test frequency ($F_{17/595} = 239.57$, $p < .0001$), and a significant interaction term ($F_{51/595} = 21.94$, $p < .0001$). Bonferroni pairwise comparisons determined that the DPOAE generated by rats exposed to 95 dB were significantly reduced relative to all other groups (color figure available online).

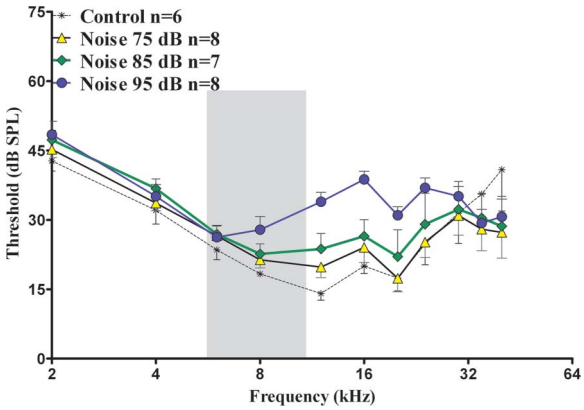

FIGURE 3. Auditory thresholds (mean ± standard error of the mean) assessed 4 wk following exposure of rats to noise treatment alone at 75, 85, and 95 dB(A) compared to untreated control subjects. The shaded area denotes the range of frequencies contained in the noise exposure. The ANOVA conducted across treatment groups failed to show a statistically significant effect of noise intensity ($F_{3/25} = 1.36$, $p > .05$), but frequency ($F_{10/250} = 30.26$, $p < .0001$) and the noise intensity by frequency interaction ($F_{30/250} = 2.78$, $p < .0001$) did reach statistical significance. Based upon the significant interaction term, a step-down analysis that compared treatment groups within the frequency band (8–20 kHz) predicted to be affected by the octave band of noise was conducted. This analysis showed a significant effect of treatment ($F_{3/25} = 7.54$, $p < .001$), frequency ($F_{3/75} = 10.39$, $p < .0001$) and a significant treatment by noise interaction ($F_{9/75} = 2.21$, $p < .05$) (color figure available online).

predicted to show NIHL (Figure 2). The extent of the loss ranged from 10 to 20 dB within the frequency band for rats receiving 95 dB, while the rats that received 85 dB noise treatment generally showed less than a 10-dB loss in the distortion product amplitude. The lowest noise treatment, 75 dB, yielded no noticeable shift in this functional measure. As there was no effect of gender upon extent of DPOAE impairment, this factor was dropped from the final statistical analysis. The ANOVA demonstrated a significant effect of noise treatment test frequency and significant interaction term. Bonferroni pairwise comparisons determined that the DPOAE generated by rats exposed to 95 dB were significantly reduced for just over a full octave between 9.0 and 19.4 kHz relative to all other groups. However, there was no significant difference in DPOAE amplitude between control subjects and those exposed either to 75-dB or to 85-dB noise.

The CAP test also conducted 4 wk postexposure demonstrated an elevation in auditory threshold among rats that received 95 dB of noise exposure (see Figure 3). Within the frequency region of the noise exposure threshold, elevations of 15–20 dB were observed in these subjects. In contrast, rats receiving 85 dB of noise showed no more than a 10-dB elevation of threshold relative to untreated controls and the rats receiving 75 dB displayed only a 5-dB threshold elevation. The ANOVA conducted across treatment groups failed to show a statistically significant effect of noise intensity, but frequency and the noise intensity by frequency interaction did reach statistical significance. Based upon the significant interaction term, a step-down analysis that compared treatment groups within the frequency band (8–20 kHz), predicted to be affected by the OBN was conducted. This analysis showed a significant effect of treatment, frequency and a significant treatment by noise interaction. Bonferroni's multiple comparisons test identified a significant difference between control subjects and those exposed to 95 dB. The highest noise exposure group, 95 dB, also demonstrated significantly poorer thresholds than either the 75 or 85 dB noise exposure group.

Figure 4 portrays the loss of OHC produced by noise exposure as a function of location along the basilar membrane of the cochlea and, thereby, by sensitivity to tone frequency. Rats receiving 95 dB OBN noise exposure showed a highly selective loss of OHC, but one limited to less than 10% within the 0.3-mm-wide band that was used as the unit for counting (Figure 5). The loss was observed in all three rows of OHC and occurred at locations corresponding to tone frequencies ranging from just under 15 kHz to 20 kHz. Rats receiving the two lower noise levels had sporadic hair cell loss that was indistinguishable from control subjects.

The effects of JP-8 exposure by itself are presented in Figures 6–8. JP-8 exposure exerted no marked effect on DPOAE amplitude for either gender or for either the 10 d (data not shown) or 4 wk postexposure test. Figure 6 presents the DPOAE data for all subjects receiving JP-8 4 wk after exposure. The data show practically no shift in DPOAE amplitude for

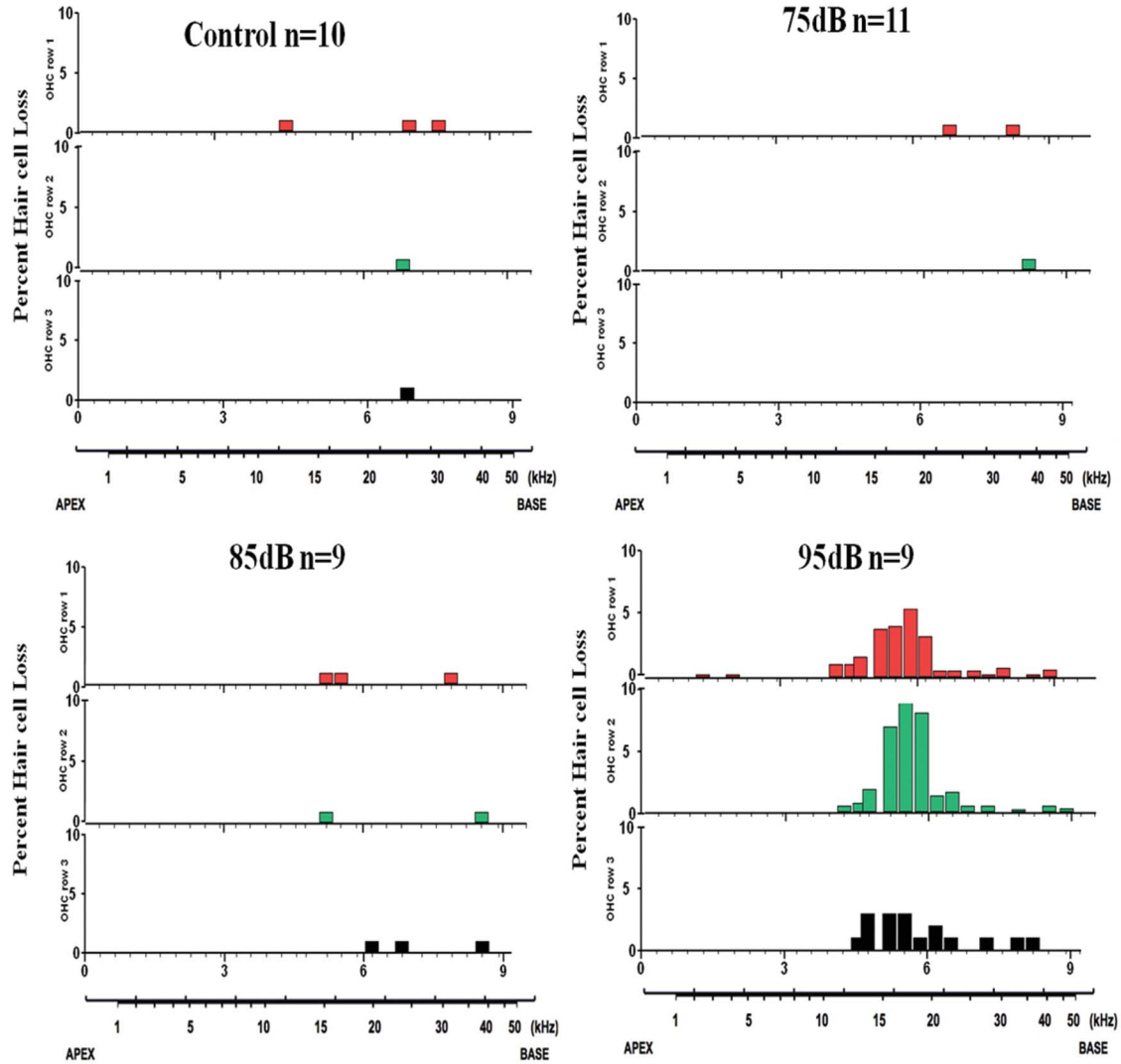

FIGURE 4. Cytocochleagrams displaying hair cell death 4 wk following exposure of rats to noise treatment alone at 75, 85, and 95 dB(A) compared to untreated control subjects (color figure available online).

any test frequency. Figure 7 portrays the effect of JP-8 exposure on auditory thresholds. Here there is no more than a 5 dB difference in auditory threshold among the groups with the largest difference from controls observed in the lowest JP-8 exposure concentration. Indeed, the highest JP-8 group resembles the control subjects more than the two other groups. Finally, Figure 8 shows the loss of OHC as a function of JP-8 exposure. There is no change in hair cell loss among treated rats compared to controls. Statistical analyses are consistent in establishing the equivalence of JP-8-treated rats and controls. In separate ANOVAs run on DPOAE and CAP data, the *F* values associated with treatment were smaller than 1, as were interactions that included the variable treatment.

The results of the combined continuous noise + JP-8 exposure study are presented in Figures 9–11. Based upon the finding that 85 dB of noise produced minimal impairment of auditory function such that the cochlear function was indistinguishable statistically from controls (Figures 2 and 3) and that no cochlear histopathology was observed (Figure 4), this noise level was utilized in a parallel study with rats that were also being exposed to 200, 750,

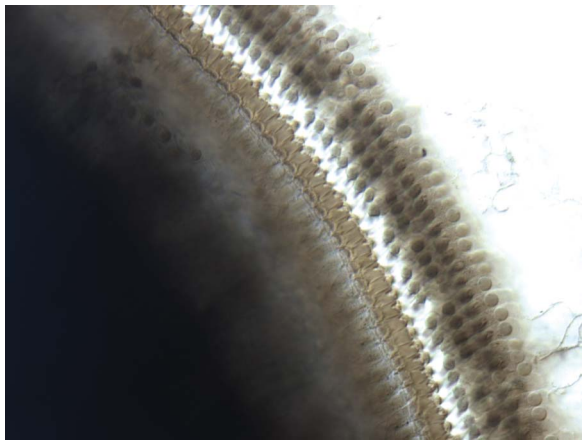

FIGURE 5. Photomicrograph (40× magnification) depicting loss of OHC in cochlear middle turn of subject exposed to 95 dB OBN (color figure available online).

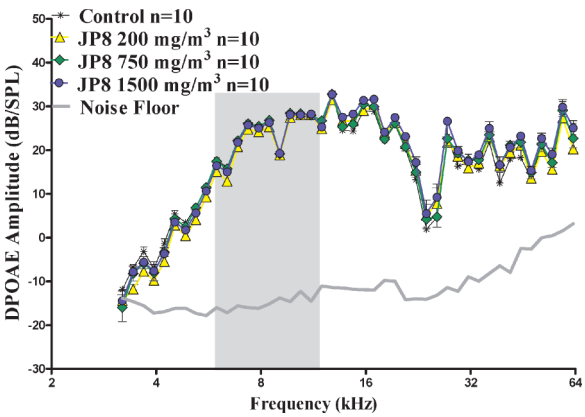

FIGURE 6. DPOAE amplitudes (mean ± standard error of the mean) among rats exposed to 200, 750, and 1500 mg/m^3 JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure (color figure available online).

or 1500 mg/m^3 JP-8 for 6 h/d, 5 d/wk for 4 wk total.

At 10 d following combined JP-8 and noise exposure, marked impairment of DPOAE amplitude was observed relative to control subjects with the effect being particularly noticeable among the 1500 mg/m^3 JP-8 + noise exposure group (Figure 9). The impairment of the DPOAE response occurred at test frequencies that coincided roughly with the lower bound of the OBN and extended to about ½ octave above the upper bound of the OBN. A repeated-measures ANOVA disclosed a significant effect of treatment, frequency, and the treatment by frequency interaction. Neither

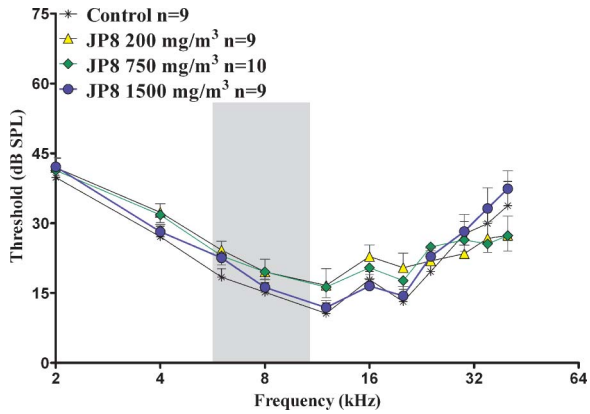

FIGURE 7. Auditory thresholds (mean ± standard error of the mean) assessed 4 wk following exposure of rats to 200, 750, and 1500 mg/m^3 JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure (color figure available online).

the effect of gender nor gender by treatment was statistically significant. Post hoc analysis by Bonferroni's multiple comparisons test showed that the rats receiving the highest JP-8 exposure dose (1500 mg/m^3) + noise differed from controls. No other significant differences were found between treatment groups.

Four weeks following the end of exposure, the extent and degree of DPOAE impairment was far more limited than at the 10 d time point (Figure 10). However, a reproducible decrease in the DPOAE response was still observed. Notably, all of the JP-8 + noise groups were impaired relative to control subjects, but did not differ from each other.

The ANOVA documented a significant effect of treatment, frequency, and the treatment by frequency interaction. Each of the fuel + noise groups showed significantly lower DPOAE responses than control. The three JP-8 + noise concentration groups did not vary significantly among themselves based upon Bonferroni's pairwise comparisons.

Pure tone auditory thresholds were elevated in the JP-8 + noise rats relative to control subjects (Figure 11). In this instance, the 1500 mg/m^3 JP-8 exposed rats showed the largest impairment although the 750 mg/m^3 JP-8 + noise subjects demonstrated similar impairment over a more limited range of frequencies. The auditory thresholds of

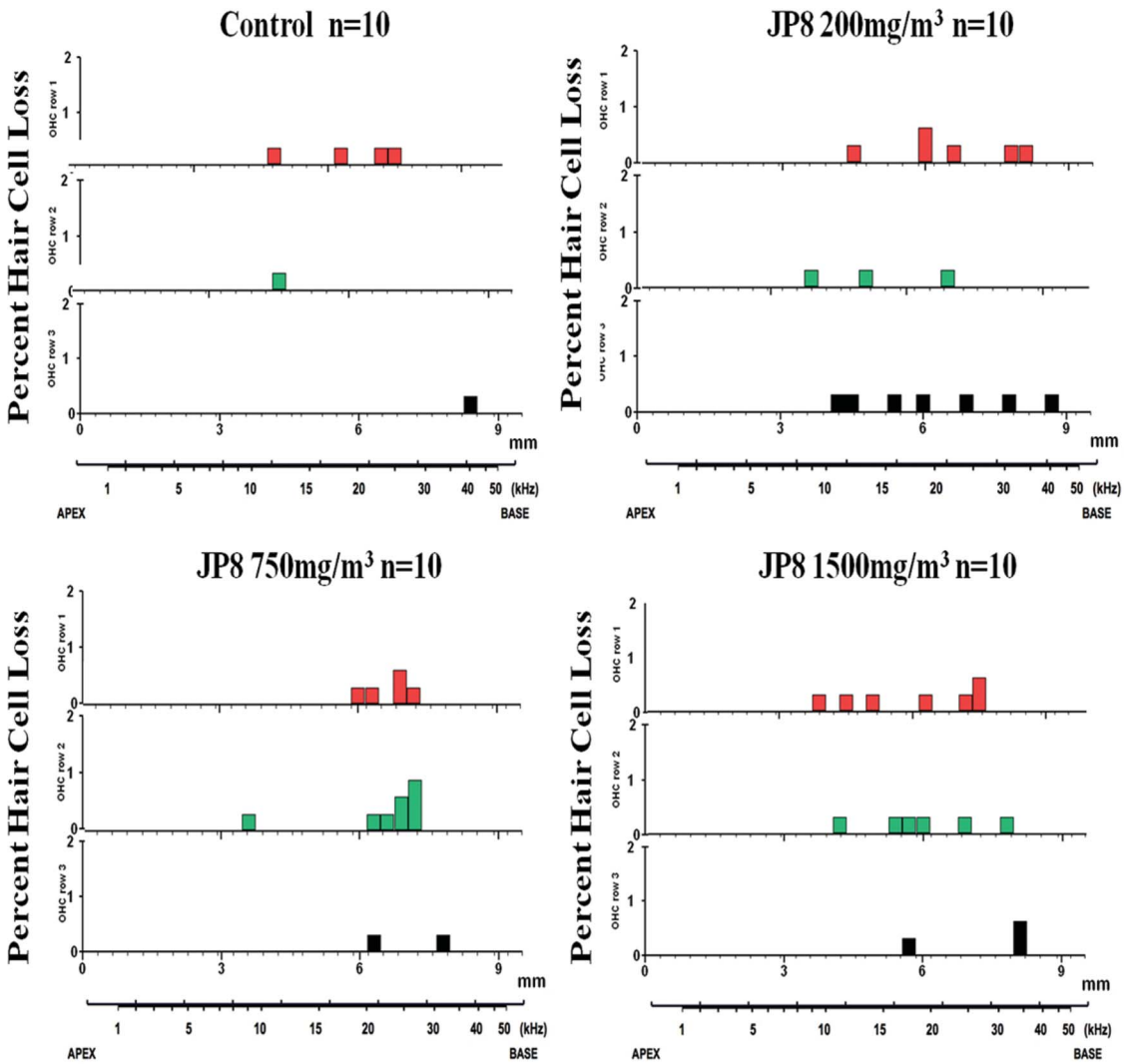

FIGURE 8. Cytocochleagrams displaying hair cell death 4 wk following exposure of rats to 200, 750, and 1500 mg/m^3 JP-8 jet fuel compared to untreated controls (color figure available online).

200 mg/m^3 JP-8 exposure + noise subjects were quite similar to that of controls. The ANOVA showed a significant effect of treatment with Bonferroni comparisons identifying a reliable difference only between the control group and the group exposed to 1500 mg/m^3 JP-8 + noise. Gender was also significant, with males having poorer hearing than females across all treatment groups. Frequency was also significant, although none of the interactions of treatment with gender or with frequency were significant.

There was sporadic OHC loss among rats exposed to the lowest two concenrations of JP-8 + noise equivalent to that seen in the control subjects (Figure 12). However, rats that received 1500 mg/m^3 JP-8 exposure + noise demonstrated a somewhat broader loss of OHC, although it was limited to no more than 1%.

The effect of JP-8 exposure on the auditory system in rats exposed to intermittent noise exposure is presented in Figures 13–15. At 10 d following exposure, a marked impairment of DPOAE amplitude was observed among rats of both genders exposed to noise and those receiving JP-8 + noise (Figure 13a and 13b). The impairment of the DPOAE response

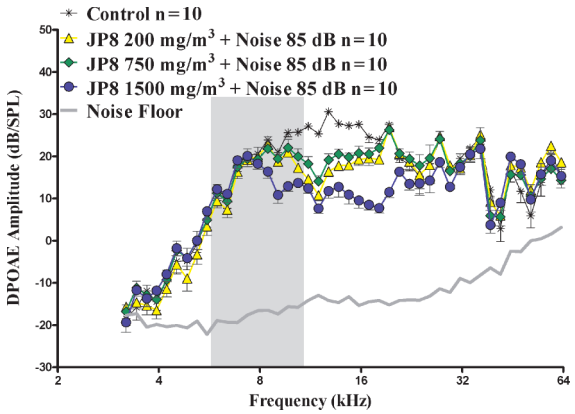

FIGURE 9. DPOAE amplitudes (mean ± standard error of the mean) assessed 10 d after exposure of rats to continuous 85-dB OBN and 200, 750, and 1500 mg/m^3 JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure. A repeated-measures ANOVA disclosed a significant effect of treatment ($F_{3/26} = 3.57$, $p < .03$), frequency ($F_{17/442} = 46.51$, $p < .0001$), and the treatment by frequency interaction ($F_{51/442} = 6.90$, $p < .0001$). Neither the effect of gender nor that of gender by treatment was statistically significant (F's < 1.0). Post hoc analysis by Bonferroni multiple comparisons test showed that the rats receiving the highest JP-8 exposure dose (1500mg/m^3) + noise differed from controls (color figure available online).

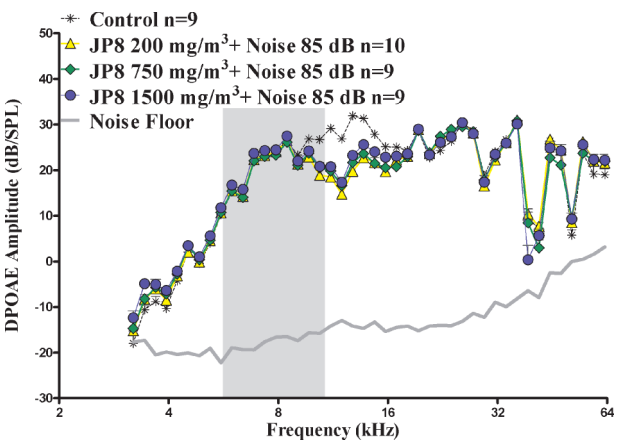

FIGURE 10. DPOAE amplitudes (mean ± standard error of the mean) assessed 4 wk after exposure of rats to continuous 85-dB OBN and 200, 750, and 1500 mg/m^3 JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure. The ANOVA documented a significant effect of treatment ($F_{3/33} = 10.62$, $p < .0001$), frequency ($F_{17/561} = 428.87$, $p < .0001$), and the treatment by frequency interaction ($F_{51/561} = 15.48$, $p < .0001$) (color figure available online).

occurred at test frequencies that coincided roughly with the lower bound of the OBN and extended to about ½ octave above the upper bound of the OBN. Four weeks following the

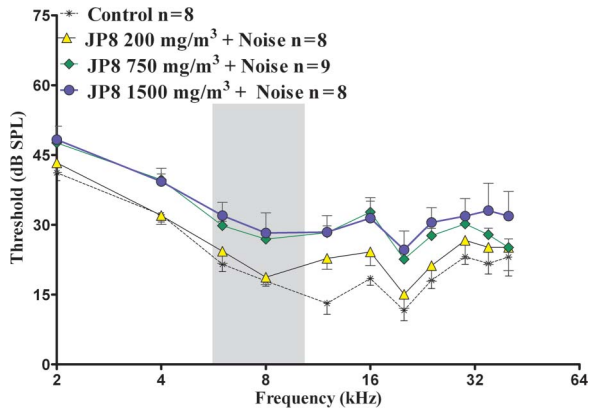

FIGURE 11. Auditory thresholds (mean ± standard error of the mean) assessed 4 wk following exposure of rats to continuous 85-dB OBN and 200, 750, and 1500 mg/m^3 JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure. The ANOVA showed a significant effect of treatment ($F_{4/29} = 2.72$, $p < .05$), with Bonferroni comparisons identifying a reliable difference only between the control group and the group exposed to 1500 mg/m^3 JP-8 + noise (color figure available online).

end of exposure, the extent and severity of DPOAE impairment are far more limited in both genders than at the 10-d time point (data not shown). However, a reproducible decrease in the DPOAE response was still observed among both the rats that received JP-8 + noise and those exposed to noise alone. This finding held true for both the male and female rats. The ANOVA conducted on the DPOAE data at 10 d postexposure showed significant effects of treatment, frequency, and the treatment by frequency interaction. Bonferroni pairwise comparisons showed that both the noise alone and the noise + JP-8 groups were significantly impaired relative to the control and JP-8 only rats. However, the two noise groups were not different statistically. Similar findings were identified 4 wk after exposure with treatment, frequency, and the interaction of these terms all meeting statistical significance. As was true at 10 d postexposure, the two noise groups differed significantly from both controls and JP-8 alone, but did not differ from each other.

Pure tone auditory thresholds were significantly impaired in the JP-8 + noise rats relative to all other groups (Figure 14). The effect was particularly noticeable at high test frequencies beyond those that would be expected to occur

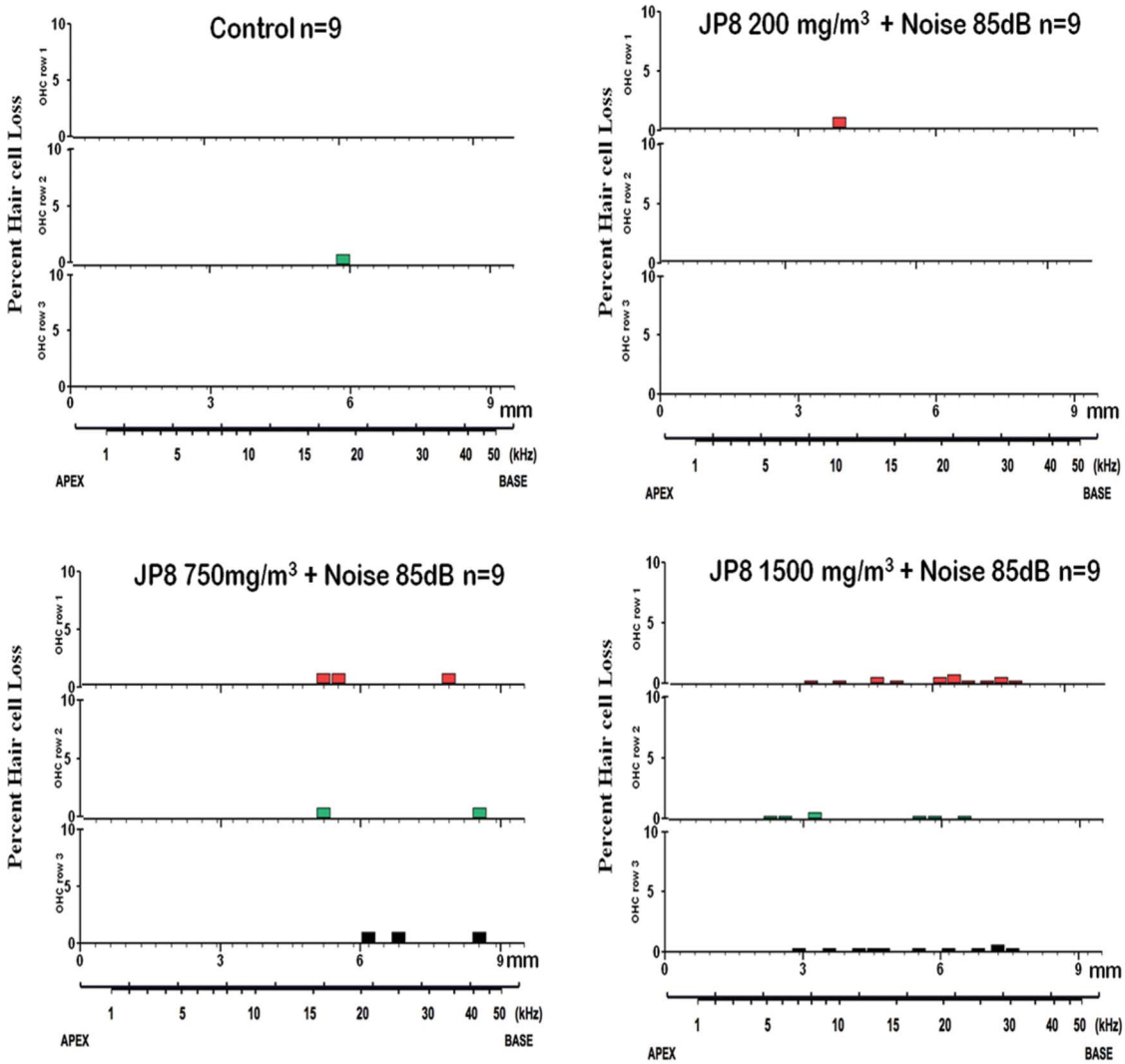

FIGURE 12. Cytocochleagrams displaying hair cell death 4 wk following exposure of rats to 200, 750, and 1500 mg/m^3 JP-8 jet fuel + 85 dB noise compared to untreated controls (color figure available online).

due to noise alone. The effect stems from a profound disruption of threshold in the male rats that received JP-8 + noise while female rats that received the combined exposure do not show greater impairment of hearing than the noise only rats. A significant concern, however, is that two male rats that had received combined treatment could not be tested. In one instance, damage to a major artery occurred during surgery leading to the death of the rat. In the other case, the round window was punctured in the process of placing an electrode onto this structure. Consequently, the CAP thresholds for the male combined exposure subjects reflect the effects seen in only three rats, while the DPOAE and the histopathology are based upon all five male rats. Analysis of auditory thresholds showed a significant effect of treatment, gender, and frequency main effects. The treatment by gender interaction did not meet statistical significance. Bonferroni multiple-comparison testing showed a significant difference between rats receiving noise + JP-8 and all other treatment groups including noise alone. In addition, the noise only rats displayed significantly elevated auditory thresholds relative to control subjects.

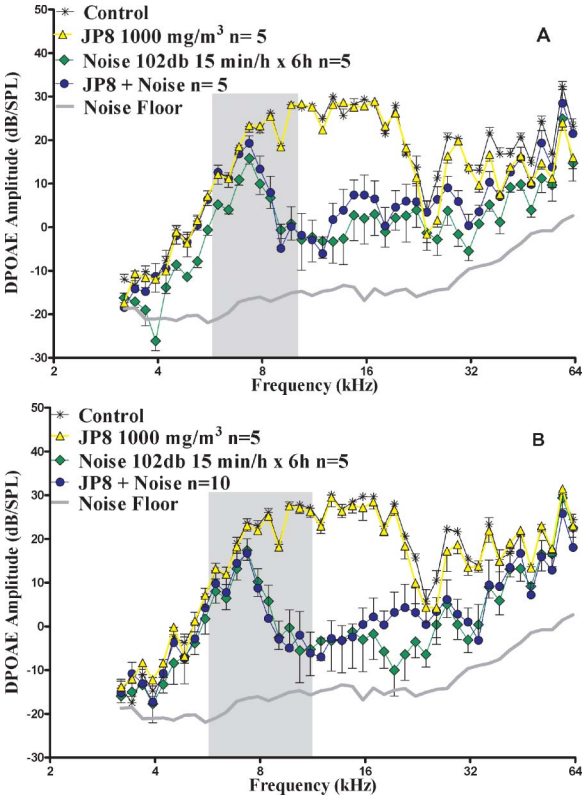

FIGURE 13. DPOAE amplitudes (mean ± standard error of the mean) 10 d postexposure among female (A) and male (B) rats exposed to 1000 mg/m^3 JP-8 + intermittent noise of 102 dB(A) for 6 h/d for 4 wk. Noise was turned on for 15 min out of each hour for a total of 90 min of exposure. Also portrayed are rats receiving either JP-8 alone, noise alone, and control subjects. The ANOVA conducted on the DPOAE data at 10 d post exposure showed significant effects of treatment ($F_{3/36} = 37.98$, $p < .0001$), frequency ($F_{17/612} = 52.37$, $p < .0001$), and the treatment by frequency interaction ($F_{51/612} = 24.29$, $p < .0001$). Bonferroni pairwise comparisons showed that both the noise alone and the noise + JP-8 groups were significantly impaired relative to the control and JP-8-only rats (color figure available online).

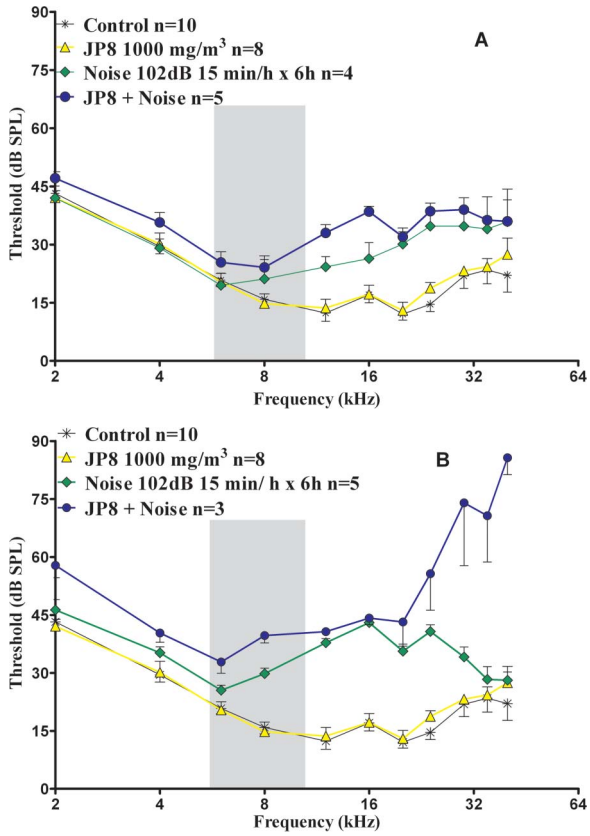

FIGURE 14. Auditory thresholds (mean ± standard error of the mean) among female (A) and male (B) rats exposed to 1000 mg/m^3 JP-8 + intermittent noise of 102 dB(A) for 6 h/d for 4 wk. Noise was turned on for 15 min out of each hour for a total of 90 min of exposure. In addition, portrayed are rats receiving either JP-8 alone, noise alone, and control subjects. Analysis of auditory thresholds showed a significant effect of treatment ($F_{3/28} = 20.79$, $p < .0001$), gender ($F_{1/28} = 6.01$, $p < .03$), and frequency ($F_{10/280} = 35.92$, $p < .0001$) main effects (color figure available online).

The effects of experimental treatment on the cochlea were also assessed by counting the number of missing/dead hair cells at the time of CAP threshold testing (Figure 14). As in all other studies the loss of OHC was sporadic and under 1% both in the control subjects and in the rats that received JP-8 alone (data not shown). There was no marked difference in OHC death between these two groups. Among those rats that received noise treatment alone, OHC loss tended to occur at locations of the cochlea that are most sensitive to sound frequencies between approximately 10–30 kHz. Solvents alone tend to produce maximal OHC loss in the middle turn of the cochlea (Lataye and Campo 1997), as well as comparable functional impairment (Crofton et al. 1994). Further, the extent of noise-induced hair cell death tended to be somewhat greater among male rats than among female rats. Finally, among male rats, those that received combined exposure to JP-8 + noise showed a broader region with missing OHC than the noise only male rats and tended to have somewhat greater rates of OHC loss. For female rats, the subjects receiving the combination of JP-8 and noise actually displayed less OHC loss than did the subjects exposed to noise alone (Figure 15).

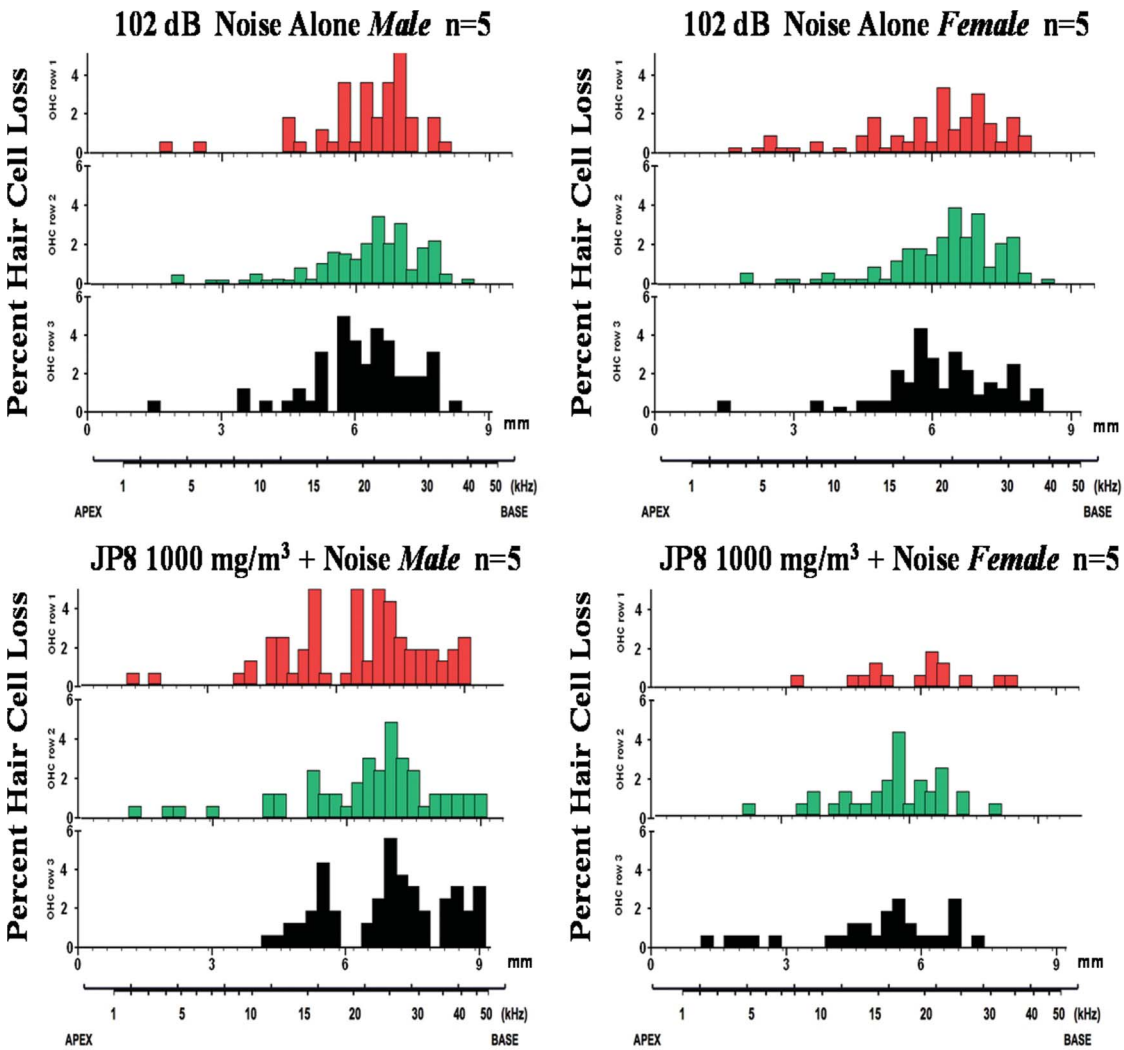

FIGURE 15. Cytocochleagrams depicting the loss of OHC among rats exposed to 1000 mg/m^3 JP-8 + intermittent noise of 102 dB(A) for 6 h/d for 4 wk. Noise was turned on for 15 min out of each hour for a total of 90 min of exposure. In addition, portrayed are rats receiving either JP-8 alone, noise alone, and control subjects (color figure available online).

DISCUSSION

These experiments focused on the vulnerability of auditory function and of cochlear integrity to exposure from JP-8 jet fuel with and without simultaneous noise exposure. The results demonstrate that JP-8 by itself is not able to disrupt cochlear function as reflected in the DPOAE, the auditory threshold, or damage to OHC, even at a concentration of 1500 mg/m^3 for 6 h/d, roughly 7.5-fold higher than the permissible human exposure level on a TWA basis. Similarly, the noise intensity used in combination with the JP-8 exposure, 85 dB for 6 h/d, exerted no significant functional or histopathological consequences. Yet when this moderate noise exposure is combined with simultaneous JP-8 jet fuel exposure, rats that received 1500 mg/m^3 of that fuel showed permanent impairment of the DPOAE response and an elevation in the CAP. There was no appreciable loss of OHC in this study.

Although it is clear that only the highest JP-8 concentration yielded a sufficiently large impairment in combination with noise to produce a reliable statistical difference, there is evidence of a trend toward impaired function in subjects exposed to 750 mg/m^3

JP-8 + noise. The LOAEL for JP-8, 1500 mg/m^3 for 6 h/d, is 7.5-fold higher than the permissible human exposure level and 750 mg/m^3 JP-8 is roughly 4-fold greater than the PEL. This finding that JP-8 + noise exposure significantly impairs the cochlea relative to rats exposed to noise alone replicates the results of a similar study conducted in this laboratory using 1000 mg/m^3 JP-8 and a higher noise exposure level (105 dB) over a 4-h/d, 5-d exposure period (Fechter et al. 2007).

The results obtained using intermittent noise of 102 dB along with JP-8 are somewhat less clear because this noise exposure level by itself produced substantial impairment of OHC function as reflected by DPOAE amplitude reduction and OHC death. Moreover, combined exposure to JP-8 + noise did not yield an increased loss in OHC function and structure compared to noise alone. However, when the neural output of the cochlea is considered, the male rats receiving JP-8 (1000 mg/m^3) + intermittent noise showed a far greater elevation of auditory thresholds than do rats that receive noise exposure alone. This elevation was seen not only in the frequency range that would be anticipated to be affected by the OBN selected, but also at higher test frequencies. The spread of impairment by chemical contaminants presented along with noise has been previously observed in the case of subacute JP-8 + noise exposure (Fechter et al. 2010).

Another feature of the enhanced susceptibility to noise observed with simultaneous JP-8 exposure is the finding that the CAP response is disrupted more reliably than is the DPOAE response. The CAP monitors the production of synchronous auditory nerve activity at the inner hair cell–spiral ganglion cell synapse, while the DPOAE response monitors OHC function. While CAP threshold sensitivity can certainly be degraded by impairment of OHC inasmuch as the OHC serve as a "gain control" for the inner hair cells, the neural elements, current evidence suggests that the inner hair cells and spiral ganglion cells may be impaired directly by JP-8 + noise.

It is not obvious why male rats appear to be more vulnerable to the enhancement of NIHL by JP-8 jet fuel exposure. However, the finding of enhanced male susceptibility was found not only in the final experiment, where intermittent noise was presented along with JP-8 jet fuel, but also in terms of auditory threshold specifically regardless of group treatment when continuous noise was paired with three different concentrations of JP-8. While the enhanced sensitivity of male rats to JP-8 + noise might reflect a true gender difference in terms of vulnerability to noise, for example, it is also possible that it might reflect toxicokinetic factors related to body fat storage rather than to a sexual dimorphism. The male and female F344 rats in our studies showed distinctly different patterns of weight gain. On average, female subjects had average body weights of 148 g at the beginning of exposure and averaged 165 g at the end of the 4-wk exposure. During the same time period, males initially averaged 187 g and averaged 243 g at the end of exposure. It is possible that the difference in body fat levels between the genders resulted in greater storage of the JP-8 fuel in male rats and, thereby, longer periods of elevated JP-8 body burdens.

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